

The University of Chicago

A STUDY OF ENZYMIC REACTIONS
CATALYZED BY PIGEON LIVER
EXTRACTS

AN EXPANSION OF A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF BIOLOGICAL SCIENCES IN
CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

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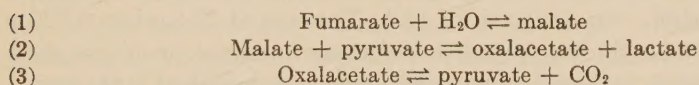
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A STUDY OF ENZYMIC REACTIONS CATALYZED BY PIGEON LIVER EXTRACTS

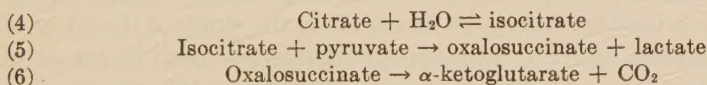
Pigeon liver is the only vertebrate tissue from which cell-free extracts capable of converting carbon dioxide into organic combination have been prepared (1). A further study of extracts from this tissue has revealed, as was expected, the presence of enzyme systems capable of catalyzing other chemical transformations. The present report is concerned with three reactions occurring in these extracts.

I. The coupled reaction between pyruvate and malate which is responsible for the fixation of carbon dioxide in cell-free extracts of pigeon liver has been investigated in more detail. As previously reported (1), the sequence may be summarized by the equations



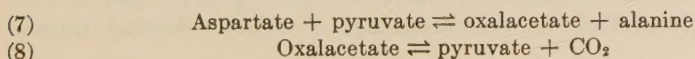
For convenience, this sequence will be referred to in the text as the reaction between pyruvate and malate.

II. A hitherto undescribed reaction between pyruvate and isocitrate to yield α -ketoglutarate and lactate has been observed. The mechanism of this reaction is believed to be analogous to that of the reaction between pyruvate and malate.



This set of reactions will be referred to as the reaction between pyruvate and isocitrate.

III. A transamination between pyruvate and aspartate has been observed and studied. The reaction mechanism is as follows:



The study of these reactions presents the following points of interest. (1) An enzymic reaction between pyruvate and isocitrate has not been described previously. (2) The reactions between pyruvate and malate and between pyruvate and isocitrate are accelerated by the presence of *either* pyridine nucleotide, diphosphopyridine nucleotide (DPN), or triphosphopyridine nucleotide (TPN). This is in marked contrast to the specific relationship between these coenzymes and the lactic (2), malic (2), and isocitric (3) dehydrogenases found in extracts of other tissues. (3) Evidence has been obtained which suggests that in pigeon liver extracts the decarboxylation of oxalosuccinate (Equation 6) is catalyzed by an enzyme similar to oxalacetate carboxylase (1). This is in contrast to the accepted view that oxalosuccinate, the hypothetical primary oxidation product of isocitrate, is spontaneously decarboxylated to α -ketoglutarate (4, 3). (4) The transamination of pyruvate with aspartate in pigeon liver extracts is catalyzed by an enzyme which appears to differ in physical properties from the transaminases previously described.

Methods

Enzyme Preparations—The pigeon liver extracts used in these experiments were prepared in the same manner as those previously employed in the study of carbon dioxide fixation (1). Pigeon liver acetone powder was extracted for 10 minutes with 8 parts of water, and the supernatant, after centrifugation, was dialyzed for 12 to 72 hours at 2° against 0.025 M phosphate, pH 7.4. Such a preparation contains fumarase, aconitase, oxalacetate carboxylase, and the various dehydrogenases described in the text.

Extracts of pigeon breast muscle were prepared from pigeon breast muscle acetone powder in the same manner.

Preparation and Assay of Pyridine Nucleotide Coenzymes—Diphosphopyridine nucleotide (DPN) was prepared from Fleischmann's bakers' yeast by the method of Williamson and Green (5). The purity of the preparation was 45 per cent as determined by hydrosulfite reduction (6).

This value was checked by use of a DPN assay method based on the glycolytic system used by Speck and Evans (7) in the study of the effect of quinine and atabrine upon the 3-phosphoglyceraldehyde dehydrogenase of rabbit skeletal muscle. Carbon dioxide production in this system is a function of the concentration of DPN. When DPN is added in amounts up to 50 γ per vessel, the relation between carbon dioxide evolution and DPN concentration is almost linear. Carbon dioxide production in the absence of added DPN is very small. The dissociation constant of the protein-coenzyme complex was found to be 3.0×10^{-5} mole of DPN per liter for the 3-phosphoglyceraldehyde dehydrogenase of rabbit skeletal muscle.

The agreement of this value with the dissociation constant of 3.16×10^{-5} mole of DPN per liter obtained by Warburg and Christian (8) for yeast 3-phosphoglyceraldehyde dehydrogenase is an indication of the validity of the assay method. No TPN was found in the DPN preparation when assayed according to Haas (9).

Part of the triphosphopyridine nucleotide (TPN) used in these experiments was a gift of Dr. Erwin Haas. Most of the work, however, was performed with a TPN preparation made by the method of Warburg, Christian, and Griese (10) as modified by Altman (11). Assay of this preparation gave a TPN content of 10 per cent (9). Its DPN content was 0.4 per cent as determined by the method outlined above.

The concentration of DPN and TPN is expressed throughout the paper in terms of the pure coenzyme as determined by appropriate assay.

Manometric Methods—Since the reactions studied in this paper all result in the liberation of carbon dioxide, manometric determination of the carbon dioxide evolution at pH 5.5 to 6.0 affords a convenient method of following the course of the reactions. An acetate buffer of pH 5.0 was added to the reaction mixtures in amounts indicated in individual experiments, and the pH of the final mixture was always between pH 5.5 and 6.0. The usual Warburg manometric technique was employed. The bath temperature was 40°, and the gas phase was air, N₂, or 95 per cent N₂-5 per cent CO₂. Frequent control experiments indicated that the oxygen consumption of the enzyme preparations was negligible at all times.¹ The substrates were added as neutral sodium or potassium salts tipped in from the side arms.

Thunberg Experiments—The Thunberg methylene blue technique was used to test the coenzyme specificity of lactic, malic, and isocitric dehydrogenases. The yeast flavoprotein, which is a component of the isocitrate system, was prepared according to the procedure of Warburg and Christian (13) up to the point of methanol precipitation. The glucose-6-phosphate system was used to study the possible conversion of DPN to TPN. For this purpose, hexose-6-phosphate and glucose-6-phosphate dehydrogenase were prepared according to the directions of Warburg and Christian (14).

Analytical Methods—Pyruvate was determined either by the carboxylase method (15) or by the colorimetric method of Friedemann and Haugen (16). Lactate was measured according to Barker and Summerson (17). α -Ketoglutarate was determined by the method of Friedemann and Haugen (16). Citrate was determined by the pentabromoacetone method of Perl-

¹ After an incubation of several hours, pigeon liver extracts consume oxygen in the presence of α -ketoglutarate. A reaction similar to the formation of malonate from oxalacetate in pig heart extracts (Vennesland and Evans (12)) is probably responsible for this delayed oxygen uptake.

man, Lardy, and Johnson (18), and the ninhydrin method of Hamilton and Van Slyke (19) was used for the estimation of α -amino nitrogen.

I. Reaction between Pyruvate and Malate

When dialyzed pigeon liver extract, together with Mn^{++} and DPN, is incubated with pyruvate and fumarate, carbon dioxide is evolved in quantities equal to the fumarate added (Fig. 1). The carbon dioxide evolution

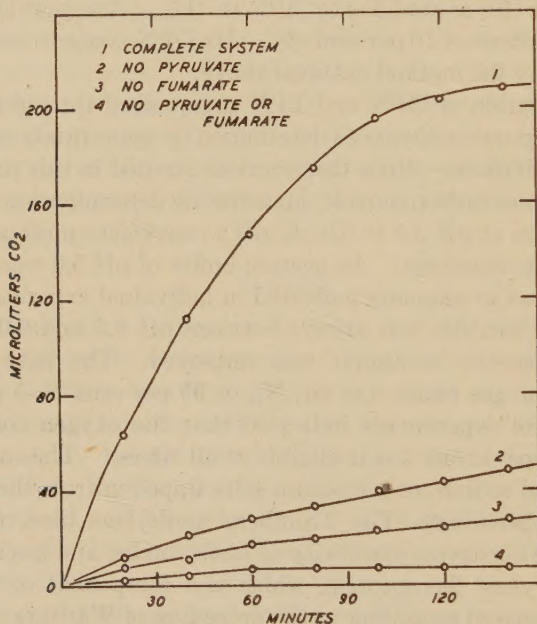


FIG. 1. The reaction between pyruvate and malate. Vessel 1 contained 1.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, at pH 5.0, 0.002 M $MnCl_2$, 5.0×10^{-5} M diphosphopyridine nucleotide, 0.004 M sodium fumarate (224 microliters), and 0.02 M sodium pyruvate (1120 microliters) in a volume of 2.4 ml. The temperature was 39° , and the gas phase was air.

is very small when either pyruvate or fumarate alone serves as a substrate, the amount obtained varying with different enzyme preparations. The behavior of *l*-malate is identical with that of fumarate, indicating that the enzyme contains an active fumarase.

These facts are in accord with the conclusion (1) that the reaction between pyruvate and malate occurs by the mechanism summarized in Equations 1, 2, and 3. The net reaction consists of a conversion of fumarate to lactate and carbon dioxide.

(9)

Fumarate $\rightarrow$ lactate + CO_2

Effect of Pyruvate Concentration—Although the pyruvate concentration does not change during the reaction, the acid being reformed in an amount equal to the quantity used, the participation of pyruvate in the reaction is reflected by the effect of pyruvate concentration on the reaction rate (Fig. 2).

Effect of Cofactors—The addition of pyruvate and fumarate to undialyzed enzyme preparations gives rise to a rapid evolution of carbon dioxide. After dialysis of the enzyme preparations, the rate of reaction between the

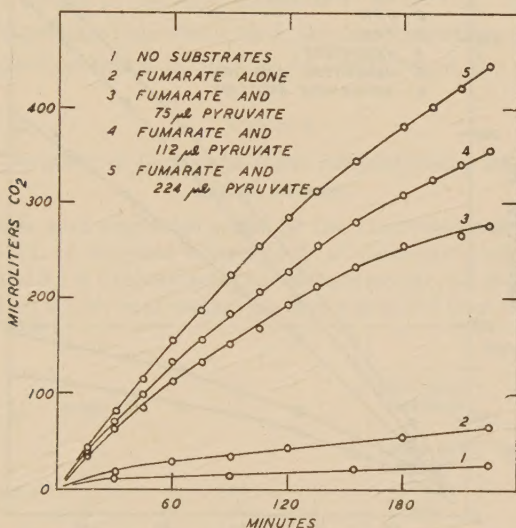


FIG. 2. The effect of pyruvate concentration on the reaction between pyruvate and malate. Vessel 1 contained 2.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, pH 5.0, 0.0004 MnCl_2 , 3.0×10^{-5} M diphosphopyridine nucleotide, and water to 3.7 ml. All the other vessels contained in addition 0.0135 M sodium fumarate (1120 microliters) and sodium pyruvate in the amounts indicated above. The temperature was 39° , and the atmosphere was 5 per cent CO_2 -95 per cent N_2 .

two substrates becomes very slow. It can be restored, however, by the addition of a boiled tissue extract.² The effect of the extract can be reproduced by the addition of Mn^{++} ions and DPN or TPN (Fig. 3). Stimulation of carbon dioxide production is caused by Mn^{++} ions alone, and either or both dinucleotides are ineffective in their absence. However, the addition of the dinucleotides plus Mn^{++} causes a more rapid evolution of carbon dioxide than that observed in the presence of the inorganic ions alone. The

² The tissue extracts were prepared by mixing 1 part of freshly ground pigeon liver or breast muscle with 1 part of water, boiling for 5 minutes, cooling, and filtering.

stimulating effect of the dinucleotides is minimal with enzyme preparations dialyzed for 12 hours and pronounced with extracts prepared by a prolonged dialysis of 48 to 72 hours. The effects of DPN and of TPN are not additive.

Since the reaction of Equation 2 is catalyzed by the lactic and malic dehydrogenases which require DPN for their action, the stimulating effect of DPN on this reaction is to be expected. However, the stimulating effect of TPN is of interest, inasmuch as the malic and lactic dehydrogenases,

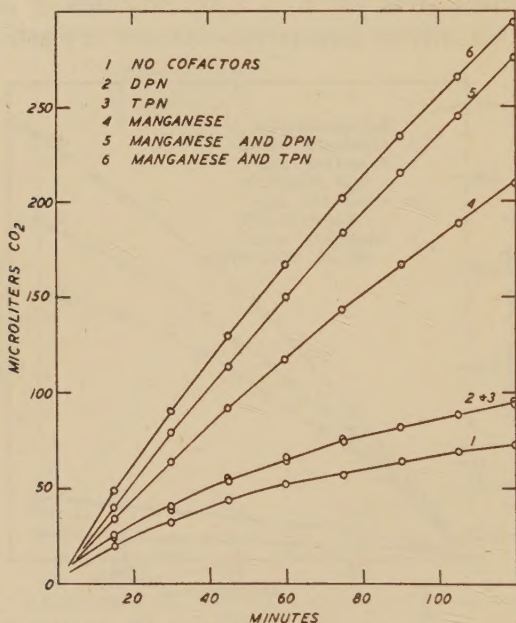


FIG. 3. The effect of cofactors on the reaction between pyruvate and malate. Vessel 1 contained 1.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, pH 5.0, 0.02 M sodium pyruvate, and 0.02 M sodium fumarate in 2.4 ml. total volume. Cofactors were added to the other vessels as indicated above in the following concentrations: diphosphopyridine nucleotide and triphosphopyridine nucleotide, 5.0×10^{-5} M; MnCl_2 , 0.002 M. All vessels were incubated in air at 39° .

when prepared from other tissues, have been found to require DPN specifically. Accordingly, an examination of the coenzyme specificities of the lactic and malic dehydrogenases of pigeon liver was carried out by the Thunberg technique. Under experimental conditions in which the coenzymes were present in concentrations less than that required for a maximum rate of methylene blue reduction, so that the rate of reduction of the dye was a function of the quantity of coenzyme present, it was found that the lactic and malic dehydrogenases of pigeon liver responded to the presence of either DPN or TPN (Table I).

While the TPN preparation used in these experiments contained small quantities of DPN, the following considerations suggest that the observed effects were due to TPN itself. Kubowitz and Ott (20) found that the dissociation constant of the protein-coenzyme complex of the lactic dehydrogenase of rat skeletal muscle was 6×10^{-6} mole of DPN per liter. While the dissociation constant of the lactic dehydrogenase of pigeon liver is not known, it may reasonably be assumed that it is not less than 10^{-6} mole of pyridine nucleotide per liter. This is the concentration of coenzyme which gives half the maximum reaction rate. In the experiment of Table I, TPN and DPN were added in concentrations of 1×10^{-5} M. In the system to which the TPN preparation was added, the concentration of DPN added in the TPN preparation was 4×10^{-7} M; *i.e.*, well below the minimal concen-

TABLE I

Coenzyme Specificity of Lactic and Malic Dehydrogenases in Extracts from Pigeon Tissues

The experiments were conducted at 25° by the Thunberg technique. System (1) consisted of 1.0 ml. of dialyzed extract, 0.02 M phosphate buffer, pH 7.4, 0.15 M NaCN, pH 7.4, and 0.1 M lithium lactate or sodium malate, all in a volume of 2.5 ml. At 0 time, 0.1 ml. of 0.1 per cent methylene blue was added from the cap.

System No.	Reduction time		
	Malic dehydrogenase	Lactic dehydrogenase	
	Pigeon liver extract	Pigeon liver extract	Pigeon breast muscle extract
	<i>min.</i>	<i>min.</i>	<i>min.</i>
1. As described above.....	35	36	60
2. Plus 1×10^{-5} M diphosphopyridine nucleotide.....	12	10	6
3. " 1×10^{-5} " triphosphopyridine ".....	14	25	17

tration giving half the maximum reaction rate. Yet the effect of TPN upon the reduction of methylene blue was from one-third to almost equal the effect of DPN. These results find their simplest explanation in the assumption that TPN as well as DPN forms an active enzyme-coenzyme complex with the lactic and malic dehydrogenases of pigeon tissues.

The effect of TPN upon lactate oxidation may also be explained by postulating the presence of an enzyme system in pigeon liver extracts capable of the rapid transformation of TPN into DPN, a reaction known to occur in yeast extracts (21). However, unpublished work by Dr. L. E. Montoya (Rockefeller Foundation Fellow) makes the occurrence of such a transformation seem improbable. Working in this laboratory, he obtained a 20-fold purification of the lactic dehydrogenase of pigeon breast muscle.

At this stage of purity, the dehydrogenase showed the same relative activity toward DPN and TPN as did the original extract. It seems unlikely that both the lactic dehydrogenase and an enzyme converting TPN into DPN would have been equally fractionated throughout the purification procedure.

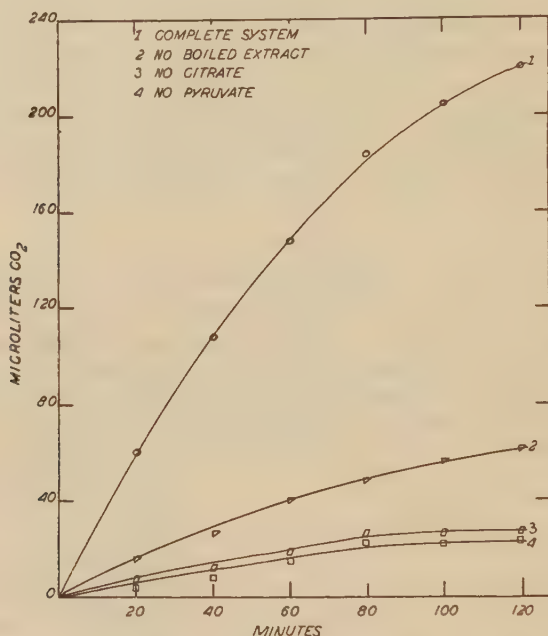


FIG. 4. The reaction between pyruvate and isocitrate. Vessel 1 contained 1.0 ml. of dialyzed pigeon liver extract, 1.0 ml. of boiled extract of pigeon breast muscle, 0.05 M acetate buffer, pH 5.0, 0.0025 M potassium citrate (224 microliters), and 0.005 M sodium pyruvate (448 microliters). The volume was 4 ml. The bath temperature was 40°, and the gas phase was 5 per cent CO₂-95 per cent N₂.

II. Reaction between Pyruvate and Isocitrate

In addition to the reaction between pyruvate and malate, pigeon liver extracts catalyze an analogous reaction between pyruvate and isocitrate. When pyruvate and citrate, together with the necessary cofactors, are incubated with pigeon liver extracts, carbon dioxide is produced in an amount equivalent to the substrate present in the smaller concentration. The carbon dioxide evolution is much less in the absence of either pyruvate or citrate or of the cofactors. A typical experiment in which the cofactors were furnished in the form of a boiled tissue extract is shown in Fig. 4.

Effect of Substrate Concentration—The optimal concentration of citrate is

about 0.0025 M. Citrate concentrations higher than this may be inhibitory and with 0.025 M citrate the reaction between pyruvate and isocitrate is almost completely inhibited. With 0.0025 M citrate, variation of the pyruvate concentration from 0.0025 to 0.0125 M does not alter appreciably the rate of the reaction.

Effect of Cofactors—The effect of boiled tissue extract on the dialyzed enzyme can be quantitatively reproduced by Mn^{++} , DPN, and TPN. As

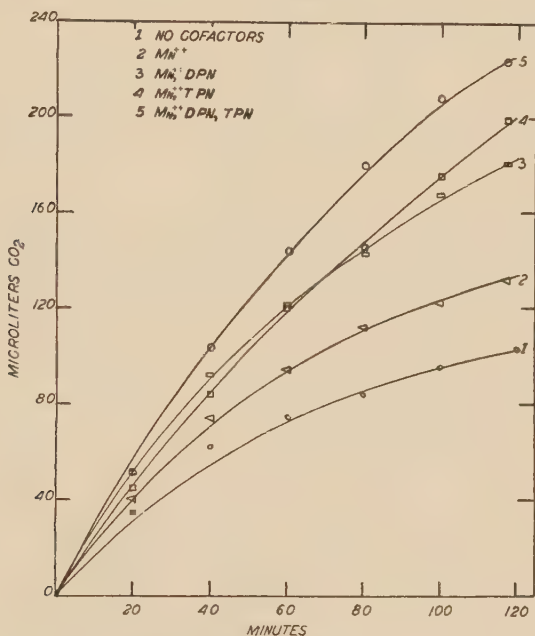


FIG. 5. The effect of cofactors on the reaction between pyruvate and isocitrate. Vessel 1 contained 1.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, pH 5.0, 0.0025 M potassium citrate (224 microliters), and 0.005 M sodium pyruvate (448 microliters). The cofactors were added to the other vessels as indicated, in the following concentrations: diphosphopyridine nucleotide and triphosphopyridine nucleotide, 3.0×10^{-5} M; $MnCl_2$, 0.005 M. The volume was 4.0 ml., the temperature 40° , and the gas phase 5 per cent CO_2 -95 per cent N_2 .

in the reaction between pyruvate and malate, Mn^{++} alone causes a large increase in the reaction rate; DPN and TPN significantly accelerate the reaction between pyruvate and isocitrate only in the presence of Mn^{++} (Fig. 5). In the experiment of Fig. 5 neither coenzyme was present in optimal concentration and the effects appear to be additive, but when DPN and TPN are added in larger amounts, no additive effect is noted.

To assure an adequate concentration of Mn^{++} , $MnCl_2$ was routinely

added in amounts larger than the quantity of citrate added, since Mn^{++} forms an un-ionized and inactive complex with citrate (22). The inhibitory effect of high citrate levels on the reaction between pyruvate and isocitrate is probably due to the combination of citrate with Mn^{++} .

Nature of Reaction between Pyruvate and Isocitrate—Analysis of initial reactants and final products in the reaction between pyruvate and isocitrate showed that for every mole of pyruvate and of citrate disappearing 1 mole each of lactate, α -ketoglutarate, and carbon dioxide was formed (Table II). The equation for the net reaction is



From the work of Martius (23), Johnson (24), and Adler *et al.* (3) it appears probable that the first step in the reaction is the transformation of citrate to

TABLE II

Chemical Changes Occurring during Reaction between Pyruvate and Isocitrate

2.0 ml. of pigeon liver extract were incubated at 40° in a reaction mixture of 4.0 ml. volume which contained 0.005 M $MnCl_2$, 3×10^{-5} M diphosphopyridine nucleotide and triphosphopyridine nucleotide, and 0.05 M acetate buffer, pH 5.0. The gas phase was 5 per cent CO_2 -95 per cent N_2 , and the incubation period was 1 hour. The substrates were added from the side arm in the amounts indicated below. (The pyruvate values are not corrected for interference on the part of α -ketoglutarate.)

The results are expressed in micromoles.

	Pyruvate	Citrate	Lactate	α -Keto-glutarate	CO_2
Initial.....	11.0	6.7	0.6	0.0	0.0
Final.....	6.5	0.4	5.9	5.6	5.0
Δ	-4.5	-6.3	+5.3	+5.6	+5.0

isocitrate by the enzyme aconitase. Isocitrate then reacts with pyruvate and yields lactate and oxalosuccinate (Equation 5). The latter is then decarboxylated to α -ketoglutarate and carbon dioxide (Equation 6).

In the data presented in Table II, lactate was determined by the Barker and Summerson method (17) (in which a copper lime reagent is used for the partial removal of pyruvate). This was considered specific for lactate and actual isolation of lactic acid was regarded as unnecessary. However, the colorimetric procedure for α -ketoglutarate (16) is not specific, and the presence of this substance was confirmed by its isolation as the 2,4-dinitrophenylhydrazone. A 100 ml. Warburg vessel was filled with 20 ml. of pigeon liver extract, 20 ml. of boiled extract of pigeon breast muscle, 2.0 ml. of M acetate buffer, pH 5.0, 2.0 ml. of 0.1 M $MnCl_2$, 2.0 ml. of 0.1 M citrate, and 2.0 ml. of 0.1 M pyruvate. After an incubation period of 90 minutes under 5 per cent CO_2 -95 per cent N_2 at 40°, the theoretical amount

of carbon dioxide had been evolved. To remove traces of pyruvate, 0.5 ml. of a yeast carboxylase preparation was added. After 5 minutes the solution was deproteinized by the addition of 0.2 volume of 20 per cent HPO_3 , centrifuged, and aerated to remove acetaldehyde. The filtrate was concentrated to 10 ml. under reduced pressure, and 10 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl were added. The hydrazone separated out immediately and, after 2 hours in the ice box, the precipitate was filtered off, dried, and weighed. 31 mg. of a substance melting at $206\text{--}207^\circ$ (corrected) were obtained (50 per cent of the quantity of α -ketoglutaric acid 2,4-dinitrophenylhydrazone demanded by theory). After two recrystallizations from 60 per cent ethanol, 14 mg. of the hydrazone were obtained which melted at 215° (corrected). An authentic sample of α -ketoglutaric acid 2,4-dinitrophenylhydrazone prepared and recrystallized in the same manner also melted at 215° (corrected). The melting point of a mixture of the two substances showed no depression. Further recrystallization from ethyl acetate and from hot water did not raise the melting point of either sample. When citrate was omitted from the reaction mixture, α -ketoglutaric acid 2,4-dinitrophenylhydrazone could not be found.

Mechanism of Reaction between Pyruvate and Isocitrate—If lactic dehydrogenase is specific for DPN and isocitric dehydrogenase is specific for TPN, it is not possible to formulate a mechanism for the direct transfer of hydrogen between pyruvate and isocitrate involving mediation by the pyridine nucleotide coenzymes alone. However, the assumption that both lactic and isocitric dehydrogenases are active with either DPN or TPN offers a plausible mechanism for hydrogen transfer between the two substrates. According to this hypothesis, either coenzyme can transfer hydrogen between pyruvate and isocitrate.

Direct test of the coenzyme specificities of the two dehydrogenases involved supported this view. As has already been shown in Table I, lactic dehydrogenase is activated by both DPN and TPN. Table III presents data which show that the isocitric dehydrogenase of pigeon liver is also active with both DPN and TPN. The approximately equal effects of DPN and TPN upon isocitrate oxidation, together with the low concentration of TPN in the DPN preparation, indicate that DPN itself can activate the isocitric dehydrogenase of pigeon liver. However, the possibility that DPN is converted into TPN was also investigated, with the glucose-6-dehydrogenase system as a test system for the formation of TPN. In previous demonstrations of the conversion of DPN to TPN in yeast (25), adenosine triphosphate was a component of the system, but since the pyruvate-isocitrate reaction proceeds in dialyzed extracts in the absence of adenosine triphosphate, none was added here. To determine the ability of the pigeon

The Thunberg experiments were carried out at 25° in a volume of 3.0 ml. System (1) consisted of 0.1 ml. of glucose-6-phosphate dehydrogenase, 0.3 ml. of yeast flavoprotein (2.5 γ of bound flavin per ml.), 0.025 M phosphate buffer, pH 7.4, 0.01 M sodium hexose-6-phosphate, and 4×10^{-5} M triphosphopyridine nucleotide. 0.1 ml. of 0.1 per cent methylene blue was added from the cap. The pigeon liver extract was incubated with the rest of the system for 15 minutes in air and 15 minutes *in vacuo* before the methylene blue was tipped in.

amount of heat-inactivated enzyme. The slight acceleration of methylene blue reduction was probably due to traces of TPN in the tissue extracts. Certainly a rapid conversion of DPN to TPN, such as would be necessary to explain the effect of DPN upon the oxidation of isocitrate in a system with pyruvate or with flavoprotein and methylene blue, was not observed.

However, other tissues, notably pigeon breast muscle, also show a similar lack of coenzyme specificity on the part of their lactic and isocitric dehydrogenases (Tables I and III). One might expect, therefore, that in such preparations the reaction between pyruvate and isocitrate should occur, since the enzyme aconitase is known to be widely distributed, and since the decarboxylation of oxalosuccinate is considered to occur spontaneously. Nevertheless, aqueous extracts of acetone powders of beef liver, pig liver, rabbit liver, pig heart, pig kidney, and pigeon breast muscle do not catalyze the reaction between pyruvate and isocitrate. Nor do they catalyze the reaction between pyruvate and malate. Since the latter reaction is inhibited by small amounts of oxalacetate which accumulate, it appears certain that the difference between extracts of pigeon liver and those of other tissues with respect to their ability to catalyze the reaction between pyruvate and malate lies in the presence of oxalacetate carboxylase in the pigeon liver extracts. An analogous inhibition of the reaction between pyruvate and isocitrate is not possible if the decarboxylation of oxalosuccinate is completely spontaneous. If it were assumed, however, that pigeon liver contains an enzyme (similar to oxalacetate carboxylase) capable of decarboxylating oxalosuccinate, the absence of this enzyme in other tissue extracts would then explain why the reaction between pyruvate and isocitrate occurs only in pigeon liver extracts. Since free oxalosuccinic acid has never been prepared, a direct examination of the rate of its spontaneous decarboxylation and of the effect of pigeon liver extract upon the process is impossible.

Effect of Partially Purified Oxalacetate Carboxylase upon Reaction between Pyruvate and Isocitrate in Extracts of Pigeon Breast Muscle—The hypothesis outlined in the previous section was tested indirectly, therefore, by adding to pigeon breast muscle extract a preparation of partially purified oxalacetate carboxylase³ free from lactic and isocitric dehydrogenases. Although

³ Evans, E. A., Jr., Francis, A. M., and Vennesland, B., unpublished results. The preparations were made from the usual pigeon liver extracts by slowly adding methanol at 0° until the concentration was 50 per cent. The precipitate was centrifuged off and discarded. The supernatant was poured into an equal volume of 0.1 M acetate buffer, pH 5.0. The precipitate was centrifuged off, redissolved in 0.025 M phosphate, pH 7.4, and dialyzed against the same buffer. Such preparations contain oxalacetate carboxylase, traces of lactic dehydrogenase, and no isocitric dehydrogenase.

incapable of catalyzing the reaction alone, this preparation caused the reaction between pyruvate and isocitrate to occur in pigeon breast muscle extracts (Fig. 6). The reaction taking place under these circumstances was identical with that which occurs in pigeon liver extracts. The presence of both pyruvate and citrate, as well as of Mn^{++} and the pyridine nucleotide coenzymes, is necessary for its occurrence. Heating of the oxalacetate carboxylase preparation causes loss of its ability to promote the reaction between pyruvate and isocitrate in pigeon breast muscle extracts. The

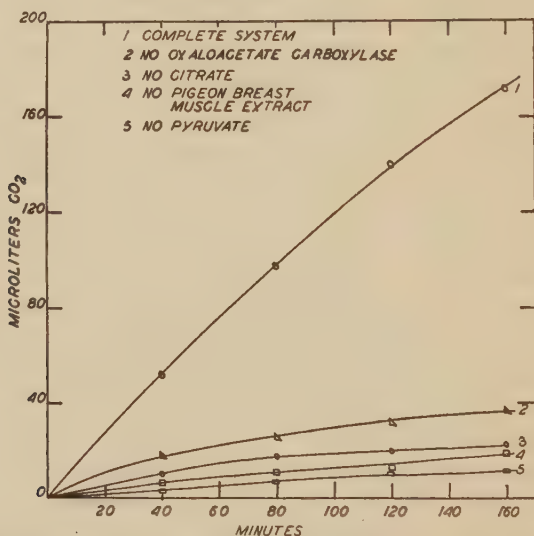


FIG. 6. The effect of partially purified oxalacetate carboxylase on the reaction between pyruvate and isocitrate in pigeon breast muscle extracts. Vessel 1 contained 1.0 ml. of dialyzed pigeon breast muscle extract, 0.5 ml. of partially purified oxalacetate carboxylase, 0.05 M acetate buffer, pH 5.0, 0.005 M $MnCl_2$, 3.0×10^{-5} M diphosphopyridine nucleotide, 3.0×10^{-5} M triphosphopyridine nucleotide, 0.0025 M potassium citrate, and 0.005 M sodium pyruvate, all in a volume of 4.0 ml. All vessels were incubated at 40° under 5 per cent CO_2 -95 per cent N_2 .

ability of partially purified oxalacetate carboxylase to catalyze the pyruvate-isocitrate reaction in pigeon breast muscle extracts roughly parallels its oxalacetate carboxylase activity.

These results indicate that pigeon liver extracts contain at least one heat-labile factor other than lactic and isocitric dehydrogenases which is essential for the occurrence of the coenzyme-linked reaction between pyruvate and isocitrate. The experimental evidence suggests that this factor is an enzyme which decarboxylates oxalosuccinate, either oxalacetate carboxylase or an enzyme similar to it.

Since di- and trivalent cations accelerate the decarboxylation of oxalacetate, it might be expected that no enzyme would be necessary for the decarboxylation of oxalacetate and that the reaction between pyruvate and malate would proceed in the absence of oxalacetate carboxylase. However, even in the presence of high concentrations of Mn^{++} ion (0.002 to 0.02 M), no reaction between pyruvate and malate is observed in the absence of oxalacetate carboxylase. Similarly, Mn^{++} ion does not catalyze the reaction between pyruvate and isocitrate, even in the presence of the proper cofactors and dehydrogenases, unless an additional heat-labile substance from pigeon liver is also present.

On the basis of the evidence outlined above, we believe that the reaction between pyruvate and isocitrate occurs in the following manner. The primary step in the reaction, the oxidation-reduction between pyruvate and isocitrate (Equation 5), is mediated by both DPN and TPN, each of which reacts with both dehydrogenase systems. If oxalosuccinate formed by the oxidation of isocitrate is not removed immediately, it then inhibits the oxidation of isocitrate in the same manner that oxalacetate inhibits the oxidation of malate (26). In pigeon breast muscle, the oxidation-reduction step proceeds readily, but the enzyme which decarboxylates oxalosuccinate is absent, and this product is removed only by non-enzymic decarboxylation. The rate of spontaneous decarboxylation is not sufficient to keep the concentration of oxalosuccinate below the level at which it inhibits the oxidation of isocitrate, and the reaction between pyruvate and isocitrate does not occur. In pigeon liver, an enzyme similar to oxalacetate carboxylase decarboxylates oxalosuccinate at such a rapid rate that its concentration in the reaction mixture does not reach an inhibitory level, and the pyruvate-isocitrate reaction goes to completion.

Oxidation of Isocitrate by Yeast Flavoprotein and Methylene Blue—However, this mechanism does not explain why isocitrate is oxidized by pigeon breast muscle extracts in the presence of yeast flavoprotein and methylene blue (3) (Table III), although isocitrate is not oxidized by these extracts in the presence of pyruvate. We have confirmed the observation of Adler *et al.* (3) that crude yeast flavoprotein is necessary for the oxidation of isocitrate by methylene blue (Table III). Since the concentration of the flavoprotein which reoxidizes reduced TPN was low in their preparation, these workers reasonably assumed that the function of the added yeast flavoprotein (which reoxidizes both TPN and DPN) was to reoxidize the TPN reduced by isocitrate.



However, it is difficult to apply this explanation to the results obtained with extracts of pigeon tissues. In these extracts, yeast flavoprotein is required

for the reduction of methylene blue in the presence of isocitrate when either DPN or TPN is present (Table III). Yet with lactate or malate as the substrate, no added flavoprotein is necessary (Table I). If it is assumed that the only function of the yeast flavoprotein is as a mediator in hydrogen transport between the pyridine nucleotides and methylene blue, then it must be concluded that the coenzymes reduced by isocitrate differ in some manner from the coenzymes reduced by lactate or malate. Since such a

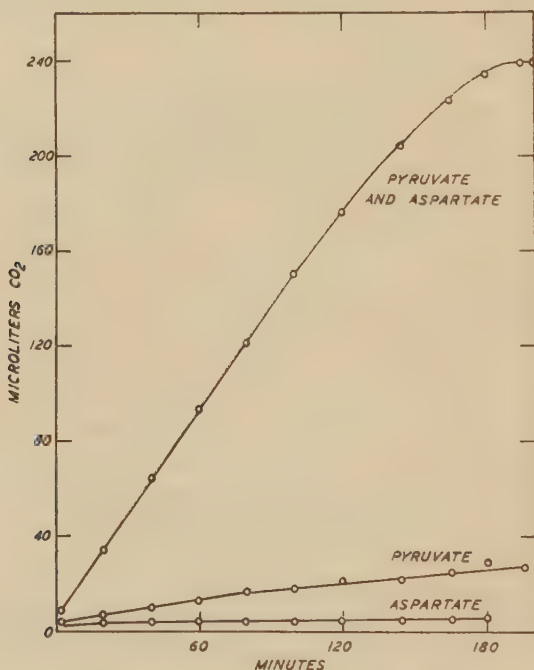


FIG. 7. The reaction between pyruvate and aspartate. All vessels contained 1.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, pH 5.0, 0.002 M MnCl_2 , and water to a volume of 2.2 ml. 0.0045 M sodium *l*-aspartate (224 microliters) and 0.0225 M sodium pyruvate (1120 microliters) were added as indicated above. The temperature was 39° and the atmosphere was air.

conclusion is unsatisfactory, we feel that in extracts of pigeon liver and breast muscle crude yeast flavoprotein plays some rôle in the oxidation of isocitrate by methylene blue other than that of a simple mediator, but we are at present unable to suggest what such an additional rôle might be. Detailed knowledge of the mechanism of isocitrate oxidation in the systems with pyruvate and with flavoprotein and methylene blue will undoubtedly resolve the apparent conflict between the two sets of observations.

III. Reaction between Pyruvate and Aspartate

When pigeon liver extract is incubated with pyruvate and *l*-aspartate, a quantity of carbon dioxide is evolved equal to the aspartate added (Fig. 7). The reaction requires Mn^{++} , but DPN and TPN have no effect on the reaction rate (Fig. 8). These results can be explained by postulating a transamination of pyruvate with aspartate to form alanine and oxalacetate,

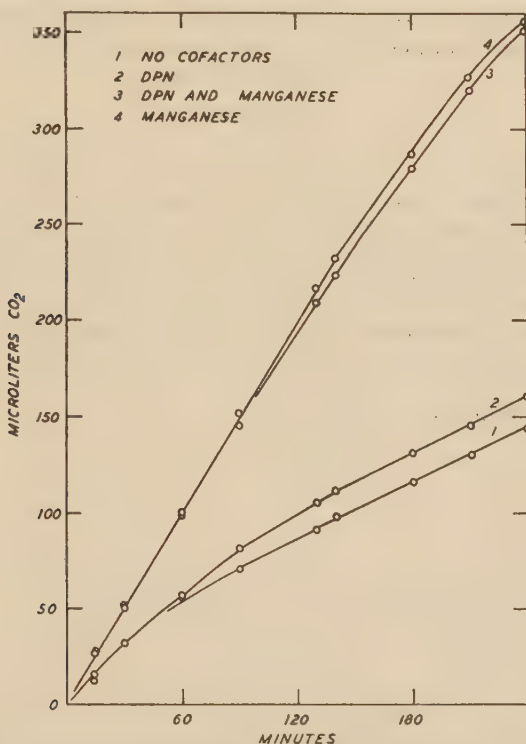
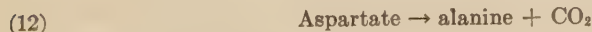


FIG. 8. The effect of cofactors on the reaction between pyruvate and aspartate. Vessel 1 contained 1.0 ml. of dialyzed pigeon liver extract, 0.05 M acetate buffer, pH 5.0, 0.02 M sodium pyruvate, and 0.008 M sodium *l*-aspartate in 2.4 ml. 5.0×10^{-5} M diphosphopyridine nucleotide and 0.002 M $MnCl_2$ were added to the other vessels as indicated. All were incubated in air at 39° .

the latter then being decarboxylated by oxalacetate carboxylase (Equations 7 and 8). The equation for the net reaction would be



The validity of Equation 12 was tested by analyzing for pyruvate and by measuring the carbon dioxide liberated by treatment with ninhydrin.

While all other α -amino acids yield 1 mole of carbon dioxide when treated with ninhydrin, aspartate forms an unstable aldehyde which spontaneously decarboxylates to liberate an additional molecule of carbon dioxide (27). Therefore, if Equation 12 is the correct representation of the reaction between pyruvate and aspartate, at the end of the reaction the carbon dioxide liberated by ninhydrin should be half the initial value, while the pyruvate concentration should be unaltered. The data of Table V indicate that this is indeed the case and thus furnish evidence for the mechanism postulated for the reaction between pyruvate and aspartate.

TABLE V

Chemical Changes Occurring during Reaction between Pyruvate and Aspartate

The reaction mixture consisted of 1.5 ml. of pigeon liver extract, 0.002 M MnCl_2 , 0.06 M acetate buffer, pH 5.0, 20 micromoles of sodium pyruvate, and 10 micromoles of sodium *l*-aspartate. The volume was 2.8 ml. After 4 hours incubation at 39° in an atmosphere of 5 per cent CO_2 -95 per cent N_2 , CO_2 evolution ceased, and the analyses were performed. The values below are corrected for the slow disappearance of pyruvate in the absence of aspartate (1 micromole per hour) and for the formation of α -amino nitrogen in the absence of any added substrate (1.5 micromoles per hour).

The results are expressed in micromoles.

	Pyruvate	Ninhydrin carboxyl CO_2	CO_2
Initial	20.0	20.0	0.0
Final	19.4	9.1	8.9
Δ	-0.6	-10.9	+8.9

DISCUSSION

Rôle of Oxalacetate Carboxylase in Coupled Reactions—In addition to the coupled enzymic reactions described in the present report, reactions between α -ketoglutarate and fumarate and between α -ketoglutarate and citrate have also been observed. These reactions are slower in rate and have not been studied in detail. The interaction between the various pairs of substrates can be explained by a general reaction scheme involving an oxidation-reduction or a transamination followed by the decarboxylation of a β -keto acid, either oxalacetate or oxalosuccinate.

The enzymic decarboxylation of oxalacetate by oxalacetate carboxylase can be demonstrated directly (1). Although a direct test is not possible in the case of oxalosuccinate, the evidence presented here suggests that oxalacetate carboxylase (or a similar component of pigeon liver extract) catalyzes the decarboxylation of oxalosuccinate. Final proof for this mechanism requires preparation of free oxalosuccinic acid. The availability of

this compound would also undoubtedly be of great assistance in understanding the conflicting data resulting from studies of pyruvate and of methylene blue as the ultimate oxidizing agents for isocitrate in the pigeon breast muscle extracts.

The reaction between pyruvate and isocitrate resembles in many ways the reaction between pyruvate and malate in which carbon dioxide is fixed into organic acid carboxyl groups, but it is unknown whether carbon dioxide fixation occurs during the course of this reaction.

Specificity of Dehydrogenases toward Pyridine Nucleotide Coenzymes—Dehydrogenases may be divided into three groups on the basis of their reactions with the pyridine nucleotide coenzymes. One group transfers hydrogen from its substrates to DPN but not to TPN. A second group transfers hydrogen to TPN but not to DPN, and a third transfers hydrogen to both coenzymes with approximately equal ease. The 3-phosphoglyceraldehyde dehydrogenase of yeast (28), the lactic and malic dehydrogenases of pig heart (2), and the alcohol dehydrogenase of yeast (28) and of mammalian tissues (29) are all examples of the first group. Glucose-6-phosphate dehydrogenase of yeast (30) and isocitric dehydrogenase of pig heart (3) are specific for TPN. The glucose (31) and *l*-glutamic (32) dehydrogenases of mammalian liver belong to the last group of dehydrogenases. Proper recognition of coenzyme specificity may be obscured by interconversion of the coenzymes in the crude enzyme preparations frequently used. Such an interconversion has been demonstrated in yeast by von Euler and his coworkers (21, 25).

Our work with pigeon tissues has led us to the conclusion that a larger number of dehydrogenases react with both DPN and TPN than has hitherto been thought. The lactic and isocitric enzymes of pigeon tissues on the one hand and of pig heart on the other offer another example of the occurrence in different tissues of dehydrogenases with different coenzyme specificity but with identical substrate specificity.

Transamination in Pigeon Liver Extracts—The presence of transaminase activity in dialyzed extracts of pigeon liver acetone powder is unexpected, since the transaminases of other tissue preparations are often destroyed by drying (33) or by dialysis (33, 34). It is probable that the transaminase of pigeon liver extracts is markedly different from those previously described. Although Braunshtein and Kritsman (35) originally reported that a wide variety of amino acids entered into reactions of transamination, Cohen (33) found that only glutamic acid, aspartic acid, and alanine undergo transamination in tissue extracts at a significant rate. It is suggested that in pigeon liver aspartate might conceivably transaminate keto acids other than those corresponding to the amino acids mentioned above, because the rapid decarboxylation of oxalacetate would drive the reaction in the direction of oxalacetate formation and the transamination of the original keto acid.

SUMMARY

Reactions occurring in cell-free extracts of pigeon liver have been studied.

1. The reaction between pyruvate and malate, which results in the fixation of carbon dioxide has been further investigated.

2. An analogous reaction between pyruvate and isocitrate has been observed and a mechanism suggested for the reaction.

3. A transamination between pyruvate and aspartate has been described.

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Addendum—Since this paper was submitted for publication, Ochoa (36) and Ochoa and Weisz-Tabori (37) have described in brief notes the preparation of oxalosuccinic acid and its enzymic decarboxylation by extracts of pig heart. The reoxidation of reduced triphosphopyridine nucleotide in the presence of Mn^{++} , CO_2 , and α -ketoglutarate indicates that, in pig heart extracts, both the oxidation of isocitrate to oxalosuccinate and the decarboxylation of oxalosuccinate to α -ketoglutarate are reversible reactions. These results are in full accord with the findings presented here.

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